DAEHAN HWAHAK HWOEJEE
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Dipeptidyl Carboxypeptidases에 의한 *S*-Hidpnryl Thioglycolyl Glycine의 가수분해

李 賢 宰
한 국 과 학 원

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S-Hippuryl Thioglycolyl Glycine: A New Chromogenic Substrate for Dipeptidyl Carboxypeptidases

Hyun-Jae Lee
The Korea Advanced Institute of Science, Seoul, Korea

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요 약. Dideptidyl carboxypeptidases와 angiotenisn-converting enzyme의 새로운 기질물질로서 thiol ester인 *S*-Hippuryl thioglycolyl glycine을 합성하였으며, 이 기질에 의한 간편하고도 예민한 효소 활성도의 정량방법을 제시하였다. 이 경우 효소반응 생성물인 thioglycolyl glycine은 반응계 중에 첨가한 5,5-dithio-*bis*-(2-nitrobenzoic acid), DTNB와 쉽게 반응하여 410 nm에서 강한 흡광스펙트럼을 갖는 5-thio-2-nitrobenzoic acid($\varepsilon_M = 1.36 \times 10^4$)을 형성함으로서 효소의 새로운 미량정량 방법으로 이용 가치가 크다고 본다.

ABSTRACT. A spectrophotophotometric assay technique is descriead for the measurement of free SH-groups in the enzyme reaction mixture. The method utilizes a new substrate, *S*-hippuryl-thioglycolyl-glycine(*S*-Hip-thioglycol-Gly) which is the basis for a convenient assay of angiotensin-converting enzyme and other dipeptidyl carboxypeptidases. This substrate contains an appropriately located thioester linkage that is hydrolyzed by the converting enzyme and other dipeptidyl carboxypeptidases. One of the products, thioglycolyl glycine, is readily measured by reaction with Ellman's reagent, 5,5′-dithio-*bis*-(2-nitrobenzoic acid), DTNB, to produce 5-thio-2-nitrobenzoic acid which has a strong absorption band at 410 nm. The method is sensitive($\varepsilon_M = 1.36 \times 10^4$ at 412 nm) and can be applied as a continuous recording with DTNB present in the enyymic reaction mixture.

INTRODUCTION

Angiotensin-converting enzyme(ACE), a dipeptidyl carboxypeptidase(DPCP ase), which catalyzes the hydrolysis of the biologically inactive decapeptide angiotensin I to the vasopressive octapeptide angiotensin II has been found in blood plasma[1,2] and in several animal tissues[3~6]. This enzyme may play akey role in the homeostatic control of blood pressure, and thus has been of great interest to many researchers who are working in the field of hypertension, particularly on the renin-angiotensin system. In the methodology to measure the enzyme activity, there have been reported a number of different techniques, but no specific simple and sensitive assay technique has

yet been demonstrated.

Partially purified enzyme preparations may be assayed biologically by the blood pressure rise method or by tissue contraction methods[7,8], or chemically by the ninhydrin reaction methods[7~9] Other methods which have been used for the assay of the converting enzyme activity include, radio-immunoassay employing ^{131}I-labelled angiotensins[10], radio activity assay using 10-(^{14}C) leu-angiotensin I [3,11] fluorophotometric assay using *O*-phthalaldehyde[9] and spectrophotometric assay measuring hippuric acid at 254 nm which is formed from the reaction with alternative substrates, Hip-Gly-Gly and Hip-His-Leu[6], These assay methods are basically acceptable but only for a limited purpose, and there are still some difficulties in getting a satisfactory result from kinetic study or other routine experiments.

In this communication we describe a simple method of assay for the converting enzyme and other related hydrolases using a new modified substrate, *S*-Hip-thioglycol-Gly. This substrate contains an appropriately located thioester linkage that is hydrolyzed to form a free thiol peptide product by the converting enzyme or by a dipeptidyl carboxypeptidase which also removes the C-terminus and penultimate amino acids as a dipeptide from peptide substrates[11~17].

A spectrophotometric technique employing this thioester in the enzyme reaction utilizes the reaction of the free thiol product with Ellman's reagent, 5, 5′-dithio-*bis*-(2-nitrobenzoic acid) to produce a strong absorption band of 5-thio-2-nitrobenzoic acid at 410 nm(E_M=1.36×10^4 at 412 nm)[18] The method is sensitive and is suitable for continuous recording with DTNB present in the enzymatic reaction mixture. The requirement of chloride ion distinguishes the converting enzyme from other dipeptidyl carboxypeptidases

EXPERIMENTAL METHOD and PROCEDURES

Enzyme Preparation. Both the angiotensin-converting enzyme and a second dipeptidyl carboxypeptidase were obtained from hog plasma by Sephadex G-200 gel filtration as partially purified forms with specific activities of 14.1 and 24.2 nmm oles per min. per mg. of protein when assayed with angiotensin I and Gly-Ala-Ala as substrates for the converting enzyme and dipeptidyl carboxypeptidase, respectively.

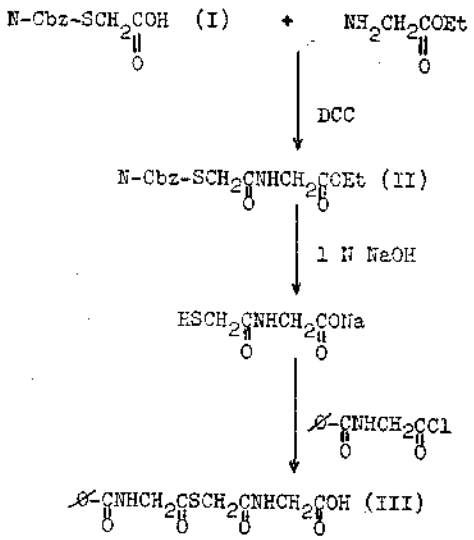
Equipment. A Cary 16 spectrophotometer equipped with a Varian G-2000 recorder and a Cary model 1626 recorder interface were used with 1.0 cm optical cells. The cell compartment was kept at 25° throughous the experiments. The absorption spectra of the substrate and the reaction product were checked by a Cary 14 recording spectrophotometer with 1 cm quartz cells.

Standard Enzyme Assay. In a 2.5 m*l* capacity optical cell, 0.25~2.5 μmoles of *S*-Hip-thioglycol-Gly and 0.96 μmoles of DTNB were placed ina final volume of 2.5 m*l* of 0.05 *M* Na-phosphate buffer, pH 7.4, and the cuvette was permitted to equilibrate to 25° in the cell compartment with or without NaCl (0.12 M). At zero time, 0.1 m*l* of test enzyme preparation was added to the cuvette and quickly but gently mixed. The changes in optical density at 412 nm were directly recorded for 5 to 30 minutes with a full scale setting of zero to 0.1. One unit of enzyme activity was expressed as one nmoles of free thiol peptide product formed per minute per mg protein.

Preparation of *S*-Hip-thioglycol-Gly, An outline for the systematic synthesis of *S*-Hip-thioglycol-Gly is shown in *Scheme* 1. Intermediate compounds from each step were characterized by m.p and elemental analysis, and were quantitized by Ellman's reaction using DTNB.

***S*-Carbobenzoxy-thioglycolic Acid.** Ten mil-

Scheme I.



iliter of freshly redistilled thioglycolic acid (150 nmoles) was mixed in 300 m*l* of 1 *N* NaOH under an ice-cooling bath, and 55 m*l* of fresh carbobenzoxy chloride was added slowly in a 30 minute period.

After the reaction, the excess of carbobenzoxy chloride was extracted twice with ether and then the aqueous solution was acidified with 6*N* HCl. The resulting white precipitate was collected on a suction funnel and washed successively with water and petroleum ether. The yield after recrystallization from ethanol-H_2O was almost theoretical. Negative test against Ellman's reagent: m. p 88~89, $C_{10}H_{10}O_4S$, Calc., C 53.1, S 14.15; Obs., C 53.17. S 14.44.

S-Carbobenzoxy Thioglycolyl Glycine Ethyl Ester (II). Fifty milimoles of glycine ethyl ester hydrochloride (7 g) was dissolvod in 500 m*l* of chloroform by adding 7.5 m*l* of triethylamine, and to the resulting clear solution 12.5 g of dicyclohexyl carbodiimide, DCC (60 nmoles) and 11.2 g of compound I (50 nmoles) were added under an ice-water cooling bath. After standing overight at room temperature the reaction was stopped by adding 1.0 m*l* of glacial acetic acid. The resulting white precipitate of dicylohexyl urea was removed by filtration and the filtrate was washed sucessively with water, 0.1 *N* HCl, 0.5 *M* Na-bicarbonate and finally with water again. The chloroform fraction was then dried over anhydrous $NaSO_4$ and was evaporated to dryness under reduced pressure. Recrystallization of the product from ethanol-petroleum ether give a fine white crystal. The yields were 90~95 % Negative test against ninhydrin and Ellman's reagent; m. p 68~69; $C_{14}H_{17}O_5NS$, Calc., C 54.1, N 4.5, S 10.03; Obs., C 54.42, N 4.55, S 9.74.

S-Hippuryl Thioglycolyl Glycine (III). Twenty milimoles of the compound II (6.22 g) was dissolved in 50 m*l* of 1 *N* NaOH, and the solution was left standing for 30 minutes at room temperature. During this stage the *S*-carbobenzoxy blocking group and carboxy ethylester group werer emoved by alkali treatment.

The solution was then neutralized to pH 7.0 with 1 *N* HCl and 5.9 g of freshly prepared hippuryl chloride (30 milimoles), which was made from hippuricacid with $POCl_3$ in acetyl chloride assolvent system, was added slowly. The resulting milky solution was then concentrated under reduced pressure, and the residue was dissolved in 20 m*l* of chloroform. After filtration of undissolved materials the solvent was removed by rotary evaporation, and the resulting product was recrystallized from ethanol-water. The yield after recrystallization was 60 %. Negative test against Ellman's reagent;

After alkaline hydrolysis of the product Ellman's color reaction showed quantitative values. m.p 135~137; $C_{13}H_{14}O_5N_2S$, Calc., C 50.32, N 9.03, S 10.32; Obs., C 50.62, N 9.01, S 10.16.

RESULTS and DISCUSSION

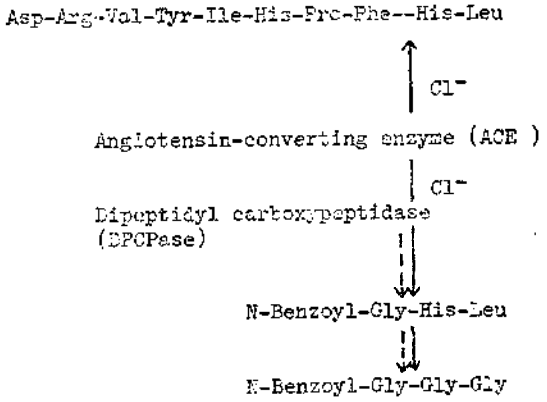
A new substrate *S*-hippuryl-thioglycolyl-gly-

cine which is the basis for a convenient assay of angiotensin-converting enzyme and other dipeptidyl carboxypeptidases was synthesized according to *Scheme* I. This thiol ester substrate was obtained as a white crystalline form (m.p 135~137) and showed no light absorption around 410 nm. The substrate is slightly soluble in cold water but is quite soluble in hot water, ethanol and buffered media. Stability of the substrate in aqueous solution was checked by quantitative Ellman's reaction using DTNB [18] and the substrate is found to be relatively stable in 0.05 *M* Na-phosphate buffer, pH 6 to 8 for a couple of days at 3 °C. The presence of hydroxylamine (1 m*M*), however, causes a steady increase in the hydrolysis of thiol ester linkage of the substrate. No hydrolysis of the substrate was found by the addition of ammonium sulfate (0.1 *M*) which might be present in the enzyme preparation.

A sequence reaction involving enzymatic hydrolysis of the thiol ester substrate in which the colored compound, 5-thio-2-nitrobenzoic acid is is produced by the reaction of a free thiol product with DTNB was shown in *Scheme* II. The difference spectrum of this colored product and the mixture of the substrate and DTNB system had a maximum at 412 nm, and a value of the molar difference extinction coefficient at the same wave length was estimated to be 1.36×10^4, in agreement with literature value [18~20].

The rate of enzymatic hydrolysis of the thiol ester substrate was measured by continuous recording of the increase in optical density at 412 nm, and was calculated based on a value of $\varepsilon_M=1.34\times10^4$. The initial velocities were proportional to the amount of enzyme as shown in *Fig.* 1. A typical experiment with angiotensin-converting enzyme is shown in *Fig.* 2. in which the enzyme activity is clearly dependent on chloride ion. This chloride ion requirement [14,16,17] may distinguish the converting enzyme from the other related peptide hydrolases as shown in *Scheme* III. When initial velocities were plotted against several different initial substrate concen-

Scheme II.



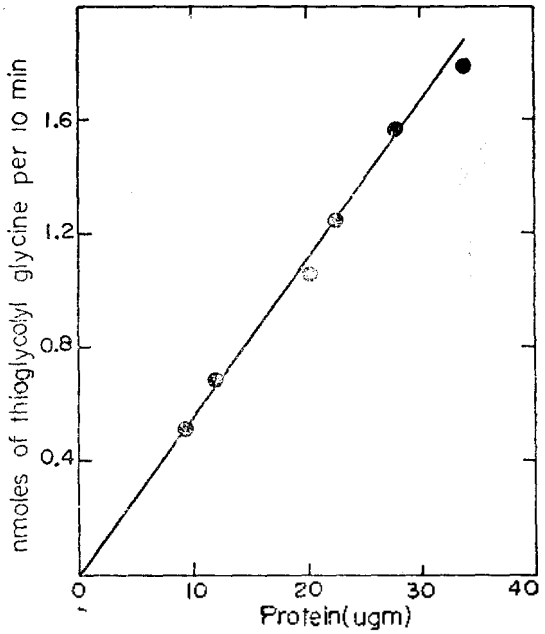


Fig. 1. Effect of protein concentration (DPCPase) on enzymic hydrolysis of *S*-Hip-thiglycol-Gly. The incubation mixture contained 2.5 μmoles of *S*-thioglycol-Gly and 0.96 μmoles of DTAB in a final volume of 2.5 m*l* of 0.05 *M* Na-phosphate buffer, pH 7.4. At zero time, 0.1 m*l* of the enzyme preparation was added to the cuvette, and the changes in optical density at 412nm were directly recorded at 25° for 10 minutes.

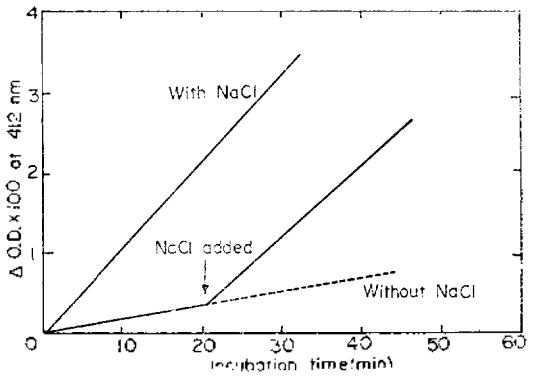


Fig. 2. The chloride ion effect on angiotensin-converting enzyme using *S*-Hip-thioglycol-Gly as a chromogenic substrate. The incubation conditions were the same as *Fig.* 1 and 3. The concentrations of the substrate and added NaCl were 4.8×10^{-4} *M* and 0.12 *M*, respectively. The changes in optical density were recorded immediately after the addition of the enzyme preparation(ACE) with a full scalesetting of zero to 0.1.

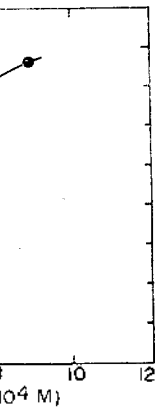
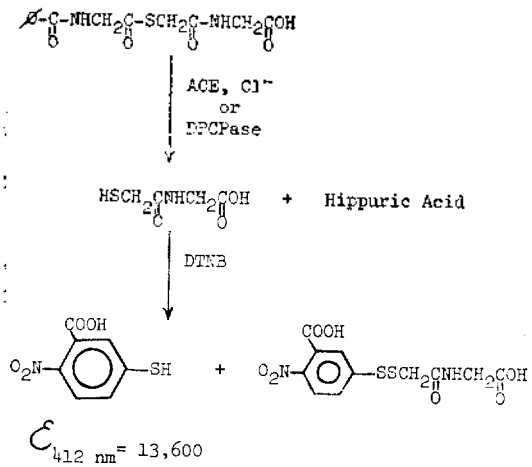


Fig. 3. Effect of substrate concetration on the activity of dipeptidyl carboxypeptidase. The incubations were carried out at 25° for 10 minutes in the presence of 7.4 μg of the enzyme preparation and 0.96 μmoles of DTNB. The substrate concentrations were varied from 1.6×10^{-4} *M* to 8.9×10^{-4} *M*.

Scheme III.



trations, an almost linear line was obtained as shown in *Fig.* 3 which resulted in some difficulty in estimating enzyme kinetic parameters. There is no K_m value in *Fig.* 3. Although as mentioned before, the substrate itself is stable in buffered media, there is an indication of non-enzymichydrolysis of thiol ester linkage of the substrate, possibly by the reaction with an aromatic thiol as a nucleophile. In fact, there has been seen a possibility of forming an aromatic thiol, 5-thio-2-nitrobenzoic acid from the reaction of DTNB with free thiols which are possibly present in the substrate as impurities, particularly at high substrate concentrations above 1.0 m*M*. Under the condition of relatively low concentrations of the substrate, below the 7×10^{-4} *M*, however, only negligible amount of non-enzymic catalysis on the substrate was observed.

In *Table* 1 we compare different methods using different substrates for assay of the enzyme activities. In a method using Hip-Gly-Gly as a substrate the enzyme activity was estimated by the amount of Gly-Gly using amino acid analyzer, JEOL model 5HA [16,17] Radioactivity assay technique [3,11] using 1-Asp-5-Ileu-10-[^{14}C]-angiotensin I as substrate was also compared with this new spectrophotometric assay method. As a result frrom *Table* 1, the new thiol ester can be used as a chromogenic substrate for the assay of both angiotensin-converting enzyme and other dipeptidyl carboxypeptidases. This new spectrophoto-

Table 1. Enzymic hydrolysis of a new thiol ester, *S*-Hip-thiogiycol-Gly.

Substrate	Conc(mM)	Enzyme activity(unit)*			
		ACE		DPCPase	
		with NaCl	No NaCl	with NaCl	No NaCl
S-Hip-thioglycol-Gly	0.67	12.4	2.2	10.2	11.5
Hip-Gly-Gly	1.0	15.3	1.8	13.6	12.2
Angiotensin I**	0.038	7.1	1.3	—	—
Gly-Ala-Ala	1.0	—	—	24.2	23.5

* One unit of the enzyme activity was defined as 1.0 nmole of the product released per minute per mg protein.

** Radioactivity assay using 1 Asp-5-Ile-10[^{14}C] Leu-angiotensin I.

metric technique employing *S*-hippuryl-thioglycolyl-glycine for the enzyme assay is very simple and sensitive. The method will be much more useful than other techniques currently available for the converting enzyme and other dipeptidyl carboxy peptidases.

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